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BRIEF COMMUNICATION

# Inhibition of putrescine biosynthesis enhanced salt stress sensitivity and decreased spermidine content in rice seedlings

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## Abstract

The effect of polyamine biosynthesis inhibitors on the salt stress response of rice seedlings was investigated. For this, DL-α-difluoromethylarginine (DFMA) and DL-α-difluoromethylornithine (DFMO), two competitive inhibitors of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), were used. The ADC and ODC are rate-limiting enzymes involved in synthesis of putrescine. The effective quantum yield of photosynthetic energy conversion ($\Phi_{PSII}$) decreased with the salt stress, and this decrease was highly significant in the treatments with DFMA and DFMO. Interestingly, addition of exogenous putrescine reduced the decline of $\Phi_{PSII}$. Putrescine content strongly decreased after one day of the inhibitor treatment. Although the content of spermidine (converted from putrescine) also showed an initial decrease in response to the inhibitors, it recovered to a similar level to that in the control after 3 d of treatment. Under the salt stress, the effect of the inhibitors on the different compounds was similar. Moreover, the addition of exogenous putrescine partially suppressed the decrease in spermidine and spermine content. A positive correlation between the spermidine and spermine content and the $\Phi_{PSII}$ was observed. The results suggest that, under salt stress, a decrease in polyamine biosynthesis and/or polyamine content has a strong negative effect on leaves and increases salt stress sensitivity.

*Additional key words*: arginine decarboxylase, chlorophyll fluorescence, ornithine decarboxylase, polyamines, spermine.

Soil salinization is one of the causes of desertification. Under conditions of excess salinity, plant growth is strongly inhibited (Boyer 1982, Yancey *et al.* 1982). Therefore, understanding the mechanisms behind salt tolerance in plants is important to be able to increase salt stress tolerance and prevent loss of vegetation in salinized soils. Polyamines, such as putrescine, spermidine, and spermine, are basic nitrogen compounds that are involved in many physiological processes in higher plants (Bouchereau *et al.* 1999, Martin-Tanguy 2001). Changes in polyamine content under various environmental stresses have been reported in many species (Flores 1991). In the model plant *Arabidopsis thaliana*, it was found that environmental stress tolerance is related to polyamine content (Yamaguchi *et al.* 2006, Cuevas *et al.* 2008, Alcázar *et al.* 2010). In our previous study, salt stress tolerance in rice seedlings increases under a low nitrogen supply (Yamamoto *et al.* 2004). In the leaf blade of rice seedlings with a high salt tolerance, the content of proline (a compatible solute) and polyamines is higher than in the leaf blades of seedlings sensitive to salt. Furthermore, in leaf blades exposed to salt stress, a correlation between the chlorophyll fluorescence parameter effective quantum yield of photosynthetic

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*Abbreviations*: ADC - arginine decarboxylase; DFMA - DL-α-difluoromethylarginine; DFMO - DL-α-difluoromethylornithine; ODC - ornithine decarboxylase; $\Phi_{PSII}$ - effective quantum yield of photosynthetic energy conversion.

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energy conversion ($\Phi_{PSII}$) and polyamine content was found suggesting that polyamines might play an important role in salt tolerance. In the present study, polyamine content in rice seedlings was controlled using DL-α-difluoromethylarginine (DFMA) and DL-α-difluoromethylornithine (DFMO), which are competitive inhibitors of enzymes involved in polyamine biosynthesis, such as arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) (Metcalf *et al.* 1978, Kallio *et al.* 1981). Leaf blades of rice seedlings were treated with inhibitors, and a change in sensitivity to salt stress was investigated by analyzing whether a decline in polyamine content due to the effect of the inhibitors was alleviated by exogenous polyamines.

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were disinfected, germinated at 30 °C in an incubator for 48 h, and grown in a chamber at day/night temperatures of 25/20 °C, a relative humidity of 60 %, a 14-h photoperiod, and an irradiance of 270 μmol $m^{-2}$ $s^{-1}$. The seedlings were grown in modified Kasugai's nutrient solution (Kasugai 1939) until the second leaf stage (about two-week-old plants). Ammonium sulfate was used as a nitrogen source in the concentration of 10 mg N $dm^{-3}$ (Yamamoto *et al.* 2004). After the appearance of the second leaf, inhibitors of polyamine biosynthesis, 2 mM DFMA and 2 mM DFMO with 0.05 % (v/v) detergent *Tween 20* (pH 7.0), were applied to the leaves using atomizer bottles. The seedlings were further treated with 100 mM NaCl added to the nutrient solution for 3 d. Experimental treatments were: I - control (only the detergent); II - the inhibitors (2 mM DFMA + DFMO); III - NaCl; IV - the inhibitors + NaCl; V - the inhibitors + NaCl + putrescine (2 mM). Each experimental plot contained 20 - 30 rice seedlings. The plants were weighed at the beginning of the experiment (0 d) and 3 d later. Intact second-leaf blades were used for measurements of chlorophyll fluorescence with a chlorophyll fluorometer (*Mini-PAM*, *Walz*, Effeltrich, Germany). The effective quantum yield of photosynthetic energy conversion ($\Phi_{PSII}$) was calculated as $(F_m'-F_t)/F_m'$, where $F_m'$ is maximum chlorophyll fluorescence yield in the light adapted state and $F_t$ is actual chlorophyll fluorescence yield in the light adapted state. The remaining leaves were frozen in liquid nitrogen and lyophilized (*FD-550R*, *Tokyo Rikakikai*, Tokyo, Japan). The freeze-dried tissue was used for extraction and determination of polyamine content by high performance liquid chromatography (HPLC) according to Flores and Galston (1982). The freeze-dried leaf tissue (0.1 g dry mass) was homogenized in 4 $cm^3$ of 0.5 M $HClO_4$ at about 65 °C for 16 - 17 h and centrifuged at 10 000 *g* for 15 min. Extracted polyamines were converted to benzoyl derivatives and separated by the HPLC system (*Shimadzu*, Kyoto, Japan) as described previously (Yamamoto *et al.* 2004). Two independent experiments with three replicates each were performed for each treatment. Differences in values between treatments were analyzed using Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$) using *KaleidaGraph 4.11* (*Synergy Software*, Reading, PA, USA). Linear regression analysis was used to analyze a relationship between $\Phi_{PSII}$ and polyamine content after 3 d of the treatment.

Marler and Mickelbart (1993) and Yamamoto *et al.* (2011) shown that a change in $\Phi_{PSII}$ is a good indicator of salt stress. In the present study, no significant differences in $\Phi_{PSII}$ were observed between the control plants and plants treated with the inhibitors only (Fig. 1). Although $\Phi_{PSII}$ decreased with the salt stress, this decline was significant only when the inhibitors were used. Interestingly, the addition of putrescine reduced the decline of $\Phi_{PSII}$. Smith *et al.* (1985) reported that a long-term treatment with DFMA and DFMO inhibits pea plant growth. However, in the present study, significant differences in plant growth between the treatments were not observed (data not shown).

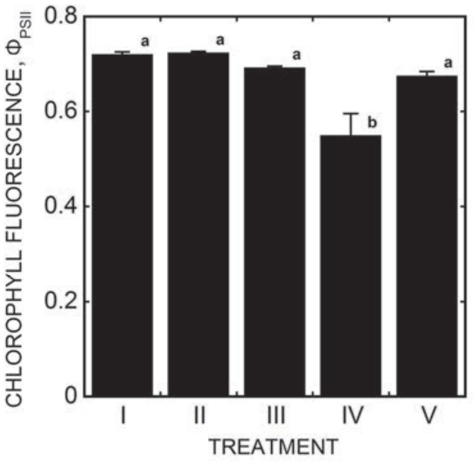

Fig. 1. Effect of inhibitors, salt stress, and putrescine on effective quantum yield of photosynthetic energy conversion ($\Phi_{PSII}$) in the 2nd leaf blades of rice seedlings after 3 d of treatment. I - control, II - inhibitors (2 mM DFMA + DFMO), III - 100 mM NaCl, IV - inhibitors + NaCl, V - inhibitors + NaCl + 2 mM putrescine. Means ± SEs, *n* = 3. Treatments marked with the same letter are not significantly different from the control (Tukey's HSD test, $P > 0.05$).

The effect of the inhibitors and salt stress treatments on the content of polyamines in leaf blades is shown in Fig. 2. Putrescine content drastically decreased after one day of the treatment with the inhibitors (Fig. 2*A*). Although the leaf surfaces were carefully washed before quantification, the measured putrescine content in treatment V was very high, possibly due to adhering exogenous putrescine. A similar decrease in spermidine content was observed after one day of the inhibitors treatment, however, its content increased after day one, reaching a similar level to the control at day three (Fig 2*B*). Spermine content, on the other hand, increased after the treatment with the inhibitors (Fig. 2*C*). The treatment with inhibitors under the salt stress caused a decrease in all compounds, and spermidine content was lowest throughout the treatment period. The addition of putrescine to the inhibitors clearly ameliorated the decline in spermidine and spermine content.

It is known that DFMA and DFMO are competitive inhibitors of ADC and ODC, respectively (Metcalf *et al.* 1978, Kallio *et al.* 1981). Since spermidine uses putrescine as a precursor, DFMA and DFMO also strongly influence spermidine content by preventing putrescine biosynthesis (Metcalf *et al.* 1978, Kallio *et al.* 1981). Higher plants synthesize putrescine *via* two pathways, through decarboxylation of ornithine or arginine. Therefore, in order to inhibit the two pathways simultaneously, the mixed solution of DFMA and DFMO was applied to the leaves. As a result, leaf blades showed a remarkable reduction of putrescine and spermidine content after one day of the treatment with the inhibitors (Fig. 2*A*,*B*).

Under the salt stress, the addition of the inhibitors accelerated the reduction of $\Phi_{PSII}$ caused by the salt stress, and after the addition of putrescine, $\Phi_{PSII}$ partially recovered. These results suggest that polyamine biosynthesis and/or polyamine content highly affect salt stress sensitivity. Lee (1997) observed that growth inhibition in rice roots due to chilling stress recovers after treatment of exogenous putrescine with DFMA and DFMO. Under salt and chilling stresses, a correlation between polyamine content in leaf blades and $\Phi_{PSII}$ has been found (Yamamoto *et al.* 2004, 2012). In the present study, a significant positive correlation was observed between spermidine content and $\Phi_{PSII}$ (Fig. 3). The same was observed between spermine content and $\Phi_{PSII}$, but not between putrescine content and $\Phi_{PSII}$ (data not shown).

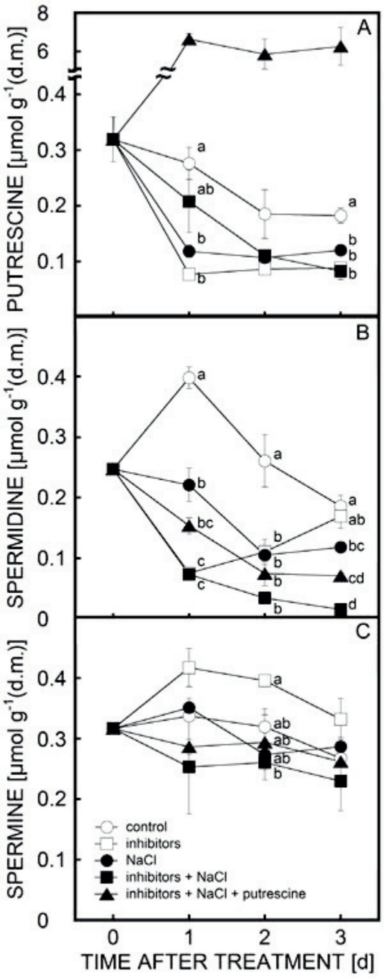

Fig. 2. The effect of inhibitors, salt stress, and exogenous putrescine on polyamine content (*A* - putrescine, *B* - spermidine, *C* - spermine) in the 2nd leaf blades of rice seedlings. Means ± SEs, *n* = 3. Treatments marked with the same letter are not significantly different from each other (Tukey's HSD test, $P > 0.05$).

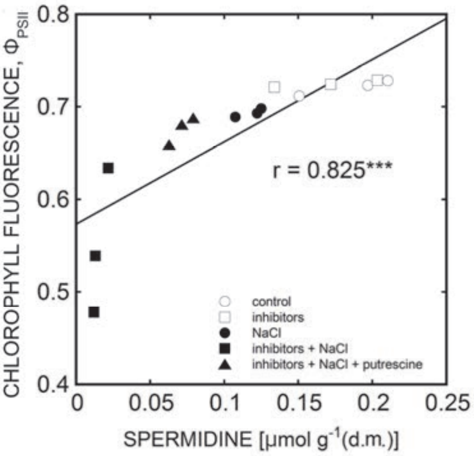

Fig. 3. Correlation between effective quantum yield of photosynthetic energy conversion ($\Phi_{PSII}$) and spermidine content in the 2nd leaf blades of rice seedlings after three days of various treatments. *** - Correlation coefficient significant at $P < 0.001$.

Roy and Wu (2002) reported that in rice plants with the overexpression of *S*-adenosylmethionine decarboxylase (a biosynthetic and rate-limiting enzyme for biosynthesis of spermidine and spermine), polyamine content increases under salt stress, and salt tolerance is improved. Moreover, it has been reported that salt tolerance of a salt-sensitive rice cultivar improves after a long-term addition of spermidine and spermine (Chattopadhayay *et al.* 2002). The above studies also showed that treatments in which a polyamine content change causes a change in plant stress sensitivity. It has been recently reported that the addition of exogenous polyamines enhances the antioxidant system of some plants, reducing environmental stress injury (Fu *et al.* 2015, Li *et al.* 2015, Mirdehghan and Rahimi 2015, Nahar *et al.* 2015). The increase in salt stress sensitivity due to the reduction of polyamines content in leaf blades and the high correlation found between spermidine content and $\Phi_{PSII}$ (this study) suggest that polyamines may protect the leaves from a decline in photosynthesis and from oxidative damage. This study clarify the positive effect of polyamines, mainly spermidine, on rice seedlings against salt-stress injury although further research is required to elucidate other functions of polyamines under salt stress conditions.

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